DEVELOPMENTAL BIOLOGY **201,** 217–229 (1998)
ARTICLE NO. DB988996

Expression of DjY1, a Protein Containing a Cold Shock Domain and RG Repeat Motifs, Is Targeted to Sites of Regeneration in Planarians

Alessandra Salvetti,* Renata Batistoni,* Paolo Deri,* Leonardo Rossi,* and John Sommerville†,[1]

**Laboratorio di Biologia Cellulare e dello Sviluppo, Dipartimento di Fisiologia e Biochimica, Università di Pisa, Via Carducci 13, 56010 Ghezzano, Pisa, Italy; and †School of Biomedical Sciences, University of St. Andrews, Bute Medical Buildings, St. Andrews, Fife, KY16 9TS, Scotland, United Kingdom*

Planarians are well-known for their exceptional regenerative abilities. This investigation focuses on the involvement of a Y-box protein, defined by the presence of a cold-shock domain, in regeneration-specific processes. Previous studies have shown that developmentally expressed Y-box proteins bind to mRNA molecules and regulate the timing of their translation. We have isolated and characterized a planarian Y-box gene, DjY1, which is specifically expressed at the site of regeneration, the blastema. DjY1 transcripts appear rapidly at the site of cutting and increase in number as the blastema grows. The timing and level of expression is similar irrespective of the orientation of the cut: in anterior, posterior, and lateral regenerative tissue. As regeneration nears completion, there is a general decrease in transcript level except in structures which are still differentiating, specifically in the auricles where new DjY1 transcripts are produced. A similarly modulated temporal pattern of expression throughout regeneration is seen in assaying the DjY1 protein. Within the population of blastemal cells, a subset of differentiating cells is specifically immunostained using antibodies to DjY1. The DjY1 protein contains a cold-shock domain and RG-repeat motifs, both of which are associated with RNA-binding properties: *in vitro* binding studies using recombinant DjY1 show that the preferred template is single-stranded RNA of heterogeneous sequence. These data provide the first direct evidence that a Y-box protein is involved in the regeneration process in planarians and implicate DjY1 in the translational regulation of differentiation-specific mRNAs.

Key Words: **planarian; Y-box protein; regeneration; gene expression; RNA binding.**

INTRODUCTION

Planarians (Platyhelminthes, Tricladida) are suitable models for the study of regenerative phenomena because of their remarkable ability to rebuild a complete individual from any small amputated fragment of their body (Montgomery and Coward, 1974). Planarian regeneration is essentially an epimorphic process which proceeds by local formation of a blastema. In the blastema, undifferentiated cells, produced by intensive cell proliferation in the region close to the wound, accumulate and differentiate (see Baguñà *et al.*, 1994, and references therein). Although dedifferentiation events have been demonstrated in planarians (Gremigni and Miceli, 1980), the generation of blastemal cells depends mainly on a permanent population of totipotent stem cells, called neoblasts. The neoblasts are considered to be the proliferative component of the planarian body, from which all specialized cell types can differentiate (reviewed in Baguñà *et al.*, 1994). As regeneration proceeds, the blastema correctly replaces the lost structures, resulting in appropriate integration into the existing body fragment.

The efficiency and reproducibility of such a process implies the presence of precise genetic controls. However, little is known of the molecular information which orchestrates the growth and subsequent shaping of the blastema in planarians. The characterization of genes involved in regeneration is a first step in understanding the molecular mechanisms involved in the activation and differentiation of the blastemal cells. Our approach to this program is to

[1] To whom correspondence should be addressed. Fax: 01334-463600. E-mail: js15@st-and.ac.uk.

0012-1606/98 $25.00

isolate genes that are known to play key roles in cellular activation, proliferation, and differentiation in other organisms.

The Y-box proteins are a family of regulatory proteins that contain an ancient nucleic acid-binding structure, the cold-shock domain (CSD; Wolffe, 1994). Whereas bacterial cold-shock proteins consist entirely of the CSD, eukaryotic Y-box proteins have an additional carboxy-tail domain which further specifies molecular interactions. The structure of the CSD has been solved and is shown to consist of a five-stranded β-barrel which presents a highly conserved set of aromatic and charged side chains on a solvent face that interacts with single-stranded nucleic acids (Schindelin *et al.*, 1994). The carboxy-tail domain is less tightly structured and shows little sequence conservation. There are, in fact, distinctly different types of nucleic acid-binding motifs in the tail domain, including arginine-rich, basic/aromatic islands (Murray *et al.*, 1992) and retroviral-type, CCHC zinc fingers (Kingsley and Palis, 1994; Moss *et al.*, 1997). A common feature is the alternation of blocks of positively and negatively charged residues, constituting a charge zipper (Wolffe, 1994) that could facilitate the formation of protein dimers and multimers. The cellular functions of Y-box proteins that have been identified include transcription regulation through binding to gene promoter elements (Wolffe, 1994) and translation repression of mRNA required at later stages of development (Matsumoto *et al.*, 1996; Sommerville and Ladomery, 1996a,b; Moss *et al.*, 1997). Several Y-box proteins share the specialized function of interacting with mRNA, yet are used in different ways to regulate translation: the cold-shock proteins of *Escherichia coli* maintain mRNA molecules in a single-stranded conformation at low temperature to facilitate their translation (Jiang *et al.*, 1997), the Y-box proteins in oocytes of *Xenopus laevis* help to prevent translation of maternal mRNA until appropriate times in development (Sommerville and Ladomery, 1996b), and the LIN-28 protein of *Caenorhabditis elegans* regulates the timing of expression of downstream genes at a posttranscriptional level (Moss *et al.*, 1997), whereas p50 in rabbit reticulocytes remains associated with mRNA through its ribosome-free and polysomal states (Davydova *et al.*, 1997). The common feature of Y-box proteins which operate in a developmental or stress-related program may be that they act as chaperones to maintain mRNA in a single-stranded conformation in readiness for expression.

In this paper we describe a Y-box protein gene, DjY1, isolated from the planarian *Dugesia japonica,* whose expression is specifically up-regulated in regenerating tissues. DjY1 transcripts are localized first in the blastema, where their level of expression relates to the progression of regeneration, and later in differentiating sensory organs, the auricles. The DjY1 protein is shown to be expressed in regeneration stages, and in blastemal tissue it is detected specifically in differentiating cells. We demonstrate that recombinant DjY1 has a strong binding affinity for mRNA sequences and that this interaction is not competed by addition of DNA or double-stranded RNA. These results conform to the view that, in the context of a developmental or stress-induced program, Y-box proteins act to chaperone mRNA molecules. We suggest that DjY1 is involved in the translational regulation of differentiation-specific genes.

MATERIALS AND METHODS

Animals

Planarians belong to the clonal strain GI of *D. japonica*, kindly provided by Dr. H. Orii. The animals were starved for 2 weeks before being used in experiments. Regenerating fragments were incubated at 18°C.

Library Screening and cDNA Sequence Analysis

Two degenerate primers, sense strand 5′-GG (T/A) AC (A/T) GT (A/G/C/T/) AA (A/G) TGGTTT-3′ and antisense strand 5′-AGT (G/A/T/C)AC(A/G) TT (A/T/G/C) GC (A/T/G/C) GC (C/T) TC-3′, were designed for the conserved amino acid residues GTVKWF and EAANVT within the CSD of most eukaryotic Y-box proteins (Wolffe *et al.*, 1992) and were used to amplify a fragment of planarian CSD cDNA by RT–PCR. Total RNA was prepared from regenerating *D. japonica*, collected 5–10 h after cutting. PCR conditions were: one cycle at 95°C for 3 min, 47°C for 90 s, 72°C for 1 min; five cycles at 95°C for 1 min, 47°C for 90 s, 72°C for 1 min; 25 cycles at 95°C for 1 min, 43°C for 90 s, 72°C for 1 min, and then 72°C for 10 min. The amplified fragment (about 200 bp) was cloned in pCR II (Invitrogen) and sequenced using the T7 DNA polymerase kit (Pharmacia). Several clones having homology with the Y-box CSD were isolated. One of these, identical at the amino acid level to other CSD clones obtained from regenerating planarians of different species (data not shown), was used to screen a λZAPII cDNA library of regenerating *D. japonica* (Orii *et al.*, 1993), at high stringency (50% formamide, 42°C). One of the positive clones, named DjY1, was sequenced on both strands.

Northern Blotting

Total RNA was obtained from whole bodies of intact and regenerating planarians, as well as from isolated blastemas, using the guanidium thiocyanate/CsCl method. Poly(A)$^+$RNA was purified using oligo(dT)-cellulose (Pharmacia). For the Northern blot analysis, RNA was electrophoresed in denaturing 1.2% agarose gels, transferred onto Hybond N$^+$ membranes (Amersham), and hybridized with ^{32}P-labeled DjY1 DNA. Filters were washed to high stringency.

Whole-Mount in Situ Hybridization

Whole mount *in situ* hybridization was performed according to Bayascas *et al.* (1997) on intact planarians and on

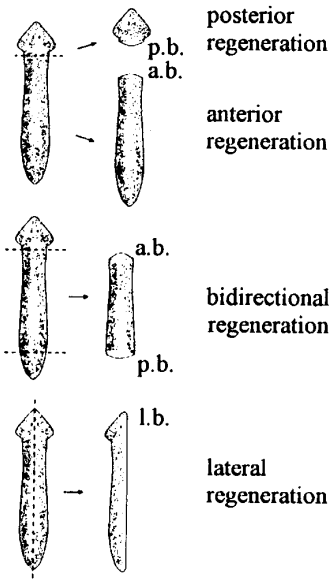

FIG. 1. Schematic representation of the cutting procedure. Dorsal view of *Dugesia japonica* amputated at different levels of the body. Different types of regeneration are shown. pb, posterior blastema; ab, anterior blastema; lb, lateral blastema.

fragments which were regenerating heads (anterior regeneration), tails (posterior regeneration), or both (bidirectional regeneration); sagittally cut (lateral regeneration) planarians were also used (Fig. 1). After *in situ* hybridization, some preparations of regenerating planarians were embedded in paraffin wax and sectioned at 6 μm. Digoxigenin-labeled riboprobes were synthesized according to standard protocols (Boehringer). The antisense probe (1106 nucleotides) was transcribed using T7 RNA polymerase from DjY1 cDNA linearized with *Eco*RI. Linearization with *Xho*I and transcription with T3 RNA polymerase generated a 619-nucleotide sense-strand RNA.

DjY1–GST Fusion Protein and Production of Antibody

A glutathione *S*-transferase fusion protein (GST–DjY1) was produced by cloning the DjY1 sequence as a *Msc*I/*Xho*I fragment (Fig. 2) into the *Sma*I/*Xho*I-digested vector pGEX4T-3 (Pharmacia). The fusion protein, corresponding to the full-length DjY1 protein less approximately half of the carboxyl tail domain, was expressed in *Escherichia coli* XL-1 Blue cells induced with 0.5 mM IPTG and grown at 30°C for 4 h in LB medium. Protein was purified by binding to glutathione–Sepharose beads (Pharmacia) and eluted with 20 mM reduced glutathione in 100 mM Tris at pH 8.0 and 100 mM KCl. Polyclonal anti-DjY1 antiserum was raised by injecting Dutch rabbits with approximately 200 μg of GST-DjY1, contained in a multiple emulsion.

North–Western Analysis

Two radiolabeled RNAs, corresponding to the 3′ end of a cDNA encoding a *Xenopus laevis* oocyte-specific β-tubulin (280 nucleotides; Ladomery and Sommerville, 1994), and to cDNA (about 1600 bp), encoding a part of *D. japonica* EF2 (translation elongation factor 2, kindly provided by Dr. H. Orii) were synthesized by *in vitro* trancription, in the presence of [α-^{32}P]CTP (Amersham). The fusion protein GST–DjY1, a nonrelated GST–RNA helicase fusion protein (Ladomery *et al.*, 1997) and bacterial protein extracts were resolved on 10% SDS–polyacrylamide gels and electroblotted onto nitrocellulose membranes. The transferred proteins were allowed to renaturate at room temperature for 30 min in TMNT binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 2 mM $MgCl_2$, 0.05% Tween 20). The blot was incubated with ^{32}P-labeled RNA for 30 min at room temperature in TMNT. The filter was washed in TMNT plus 0.2 M NaCl; afterward more stringent conditions were applied by extensive washes in TMNT containing 2.5 M NaCl.

RNA Binding Competition Assays

The binding of the sense-strand EF2 probe to GST–DjY1 in solution was competed by prior addition of various polynucleotides (Marello *et al.*, 1992). Reaction mixtures (0.1 ml) containing TMNT binding buffer, 2 μg of GST–DjY1, and 0.1 μg of probe were incubated for 30 min at 20°C in the presence of 10-, 100-, and 1000-fold excess of competitors: poly(G), poly(A), poly(C), poly(U), poly(A,G), poly(A,U), poly(A,C), poly(C,U), poly(A):poly(U), yeast tRNA, herring sperm ssDNA, poly(dI:dC), and poly(dG):poly(dC). Homopolymers and synthetic heteropolymers were purchased from Sigma Chemicals. RNA–protein complexes were separated from unbond RNA by trapping on nitrocellulose filters (0.45 μm; Schleicher and Schull). The filters were washed three times with 1 ml of TMNT binding buffer and radioactivity was determined by scintillation counting. Background controls were performed in the absence of GST–DjY1.

Western Blot Analysis

Protein extracts (40 μg) from intact planarians and isolated blastemas, taken at different stages of regeneration, were separated by SDS–PAGE. The gels were electroblotted onto nitrocellulose membranes and probed with a 1:2000 dilution of anti-DjY1 antiserum. Antibody binding was detected using an ECL chemiluminescence system (Amersham International). Protein concentration was assayed using a Bio-Rad assay system.

Immunocytochemistry

Intact planarians and isolated blastemas were dissociated into individual cells (macerates) according to the protocol described by Baguñà and Romero (1981). Cell suspensions

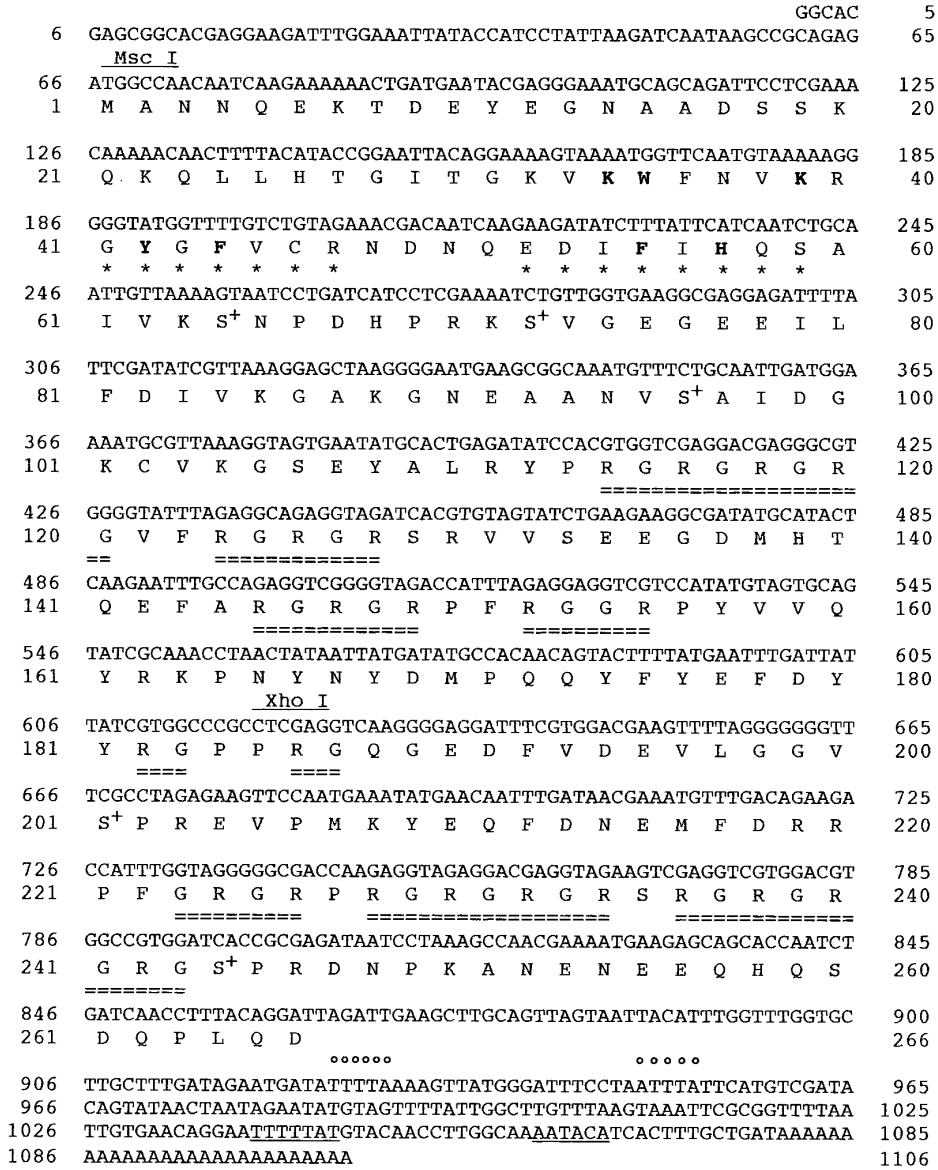

FIG. 2. Nucleotide sequence of DjY1 cDNA and its predicted amino acid sequence. Amino acid residues 31–96 comprise a cold shock domain (CSD). The conserved residues within CSDs which are believed to contact single-stranded polynucleotides are shown in bold. RNP1- and RNP2-like motifs are indicated by asterisks. The regions consisting of RG-repeats are indicated by double-dashed underlining. Amino acid residues that are potential sites of phosphorylation are followed by a plus sign. The 5′ UTR contains two in-frame stop codons at positions 45–47 and 54–56. The 3′ UTR is rich in A + T residues and contains two potential instability elements (°). The cytoplasmatic polyadenylation element (CPE) and the putative polyadenylation sequence are underlined. The two restriction sites *Msc*I and *Xho*I, used to obtain DjY1 recombinant protein, are shown. Databank Accession No. X99748.

were placed on glass slides and air-dried. Immunostaining was carried out after permeabilization with 0.1% pepsin (Bueno *et al.*, 1995), using a 1:50 dilution of the anti-DjY1 antiserum. The secondary antibody was a 1:100 dilution of peroxidase-conjugated goat anti-rabbit and the color reaction was developed using 0.5 mg/ml 3,3′-diaminobenzidine, 0.01% hydrogen peroxide in phosphate-buffered saline.

RESULTS

Isolation and Sequencing of the Planarian Y-Box Gene DjY1

In order to identify Y-box genes in planarians, a *D. japonica* cDNA library was screened with a specific probe

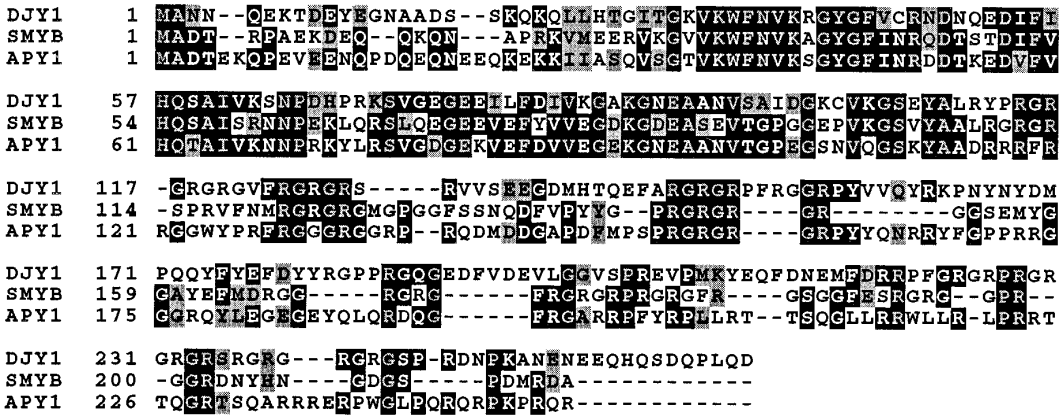

FIG. 3. Comparison of the deduced amino acid sequence of DjY1 with those of other Y-box proteins of the invertebrate subgroup: *Schistosoma mansoni* (SMYB, Accession No. U39883) and *Aplysia californica* (APY1, Accession No. U02684). The Y-box domains of SMYB and APY1 show the highest scores of similarity to DjY1 ($p = 1.6 \times e^{-46}$ and $1.1 \times e^{-39}$, respectively), using the BLASTX program. Black backgrounds indicate identical residues and gray backgrounds indicate conserved residues. Multiple alignment was obtained with the PILEUP program in the GCG package.

obtained by RT–PCR, using degenerate oligonucleotides. This probe, 198 bp long, corresponds to most of the CSD region that is highly conserved in all Y-box proteins (Wolffe, 1994). Screening led to the isolation of several positive clones, one of which (DjY1) was completely sequenced (Fig. 2). The DjY1 insert is 1106 bp long and contains an open reading frame (ORF) encoding a 266-amino-acid putative Y-box protein, DjY1, with an estimated molecular weight of 30.4 kDa. The ORF starts at an AUG initiator codon at position 66, contained within a sequence conforming to the translation start-site consensus (Kozak, 1987). The major features of the primary structure relate to the identification of two distinct potential RNA-binding domains.

The DjY1 CSD possesses the characteristic amino acids (K34, W35, Y42, F44, F55, H57) which are believed to make contact with bound nucleic-acid: these residues are highly conserved within the Y-box proteins (Schindelin *et al.*, 1994). The last four of these residues are contained in RNP-1- and RNP-2-like motifs (Landsman, 1992; Oubridge *et al.*, 1994; Schindelin *et al.*, 1994), found in RNA-binding proteins of the RRM family (Burd and Dreyfuss, 1994; Siomi and Dreyfuss, 1997). In particular, the RNP-1 hydrophobic core sequence GYGFVCR is well conserved and shows the characteristic Y-box substitution of an A to an R residue in the last position.

Although the CSD of DjY1 has all of the usual structural features, the carboxy-tail domain presents a distinctive primary structure. It is marked by the presence of numerous RG repeats, arranged in blocks and scattered throughout the domain (Fig. 2). Similar repeats have been described in diverse types of RNA-binding proteins, for instance in the snRNP core protein Sm-D1 (Rokeach *et al.*, 1988) and in the 40S ribosomal RNA protein S2 (Suzuki *et al.*, 1991). Furthermore the presence of RG repeats in DjY1 is not unique among Y-box proteins: a similar arrangement of amino acid sequences is found in ApY1 of *Aplysia californica* (Skehel and Bartsch, 1994), in SmYB of *Schistosoma mansoni* (Franco *et al.*, 1997), and in Yps of *Drosophila melanogaster* (Thieringer *et al.*, 1997). In fact, ApY1 and SmYB are the proteins most similar to DjY1, showing close homology in the CSD in addition to motif similarities in the tail domain (Fig. 3). We propose that the four RG-repeat proteins constitute a distinct subfamily of invertebrate-type RNA-binding Y-box proteins.

As described for other Y-box proteins (Sommerville and Ladomery, 1996a), several consensus motifs for protein kinases are distributed through the DjY1 sequence. These include three casein kinase II (CK2) sites, two cAMP kinase sites, and two cell division kinase sites and suggest that the activity of DjY1 could be modulated by phosphorylation.

The 3′ untranslated region (UTR) of DjY1 shows several interesting features that relate to its stability and regulation of translation. A putative cytoplasmic polyadenylation element (CPE), (TTTTTAT; Paris and Richter, 1990), is located 15 nucleotides upstream of the AATACA polyadenylation signal and may function in the control of translation of DjY1 mRNA. The 3′ UTR upstream of the CPE is A+T rich (71%) and also possesses two $A(U)_nA$ instability elements (Shaw and Kamen, 1986) at positions 924 and 949 (Fig. 2). The significance of these potential regulatory elements on the metabolism of DjY1 mRNA *in vivo* is not known.

Temporal and Spatial Expression Pattern of DjY1 Transcripts during Regeneration

Both regenerating and intact individuals were examined for DjY1 expression by whole-mount *in situ* hybridization. The types of cut used to produce regeneration are outlined

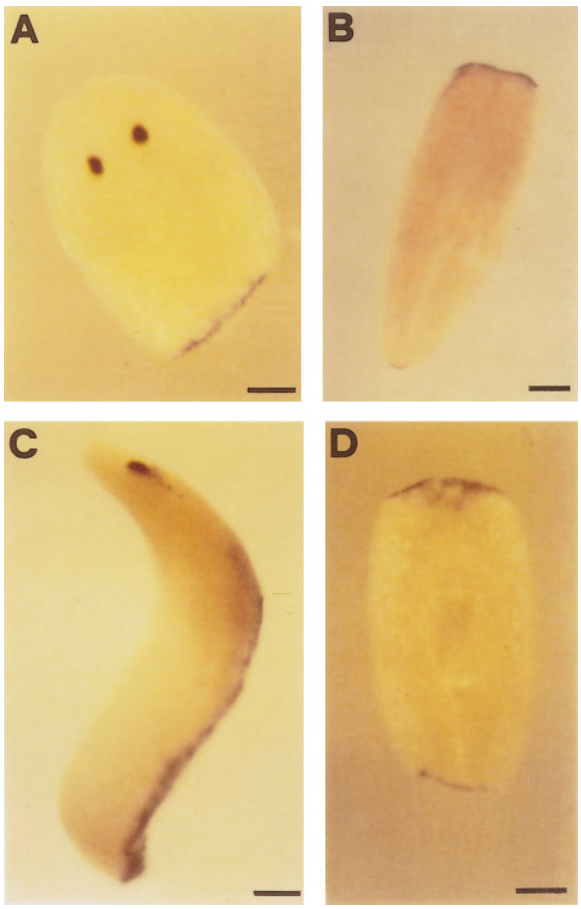

FIG. 4. Expression of DjY1 mRNA in early stages of regeneration, visualized by whole-mount *in situ* hybridization. Regenerating fragments at 3 h after cutting show DjY1 expression localized to the wound region. The expression pattern does not show significant differences in the different types of regeneration: (A) posterior, (B) anterior, (C) lateral, (D) bidirectional. Scale bars represent 400 μm.

in Fig. 1. The first localized transcripts of DjY1 are detected at very early stages of regeneration, when a thin epithelial layer has just covered the wound area. Because we observe a hybridization signal at the wound surface within a few hours of cutting (Fig. 4), it would appear that the DjY1 gene is activated almost immediately. No differences in the expression pattern have been found between posterior, anterior (cephalic), lateral, and bidirectional regenerations (Figs. 4A–4D).

As the blastema grows, the expression of DjY1 in regenerating regions increases in amount. The increase can be seen in comparing the hybridization signal in the blastema at 1 day (Fig. 5A) with that at 4 days (Fig. 5B) after cutting. Examination of many preparations indicates that the maximal level of expression of DjY1 transcripts occurs at 4 days after cutting. Wax sections of hybridized planarians show the signal to be distributed throughout the area of the blastema and to terminate abruptly at the amputation boundary (Figs. 5C and 5D).

At 5–6 days after cutting, differentiation of the missing structures becomes evident in the cephalic blastema with eyespot formation (Coward, 1968). At this time, we observe that the level of expression of DjY1 transcripts decreases. A similar decrease occurs independently of the orientation of cut (Figs. 5E and 5F).

An unique pattern of hybridization appears in the anterior blastema 13 days after cutting. Here the signal is concentrated in the region in which auricles are differentiating (Figs. 5G–5I). The auricles contain ciliary chemoreceptor cells and are considered to be the principal olfactory organs of planarians (Farnesi and Tei, 1980). These structures differentiate late in cephalic regeneration, their modeling and the presence of full pigmentation representing anatomical completion of the planarian head.

We failed to observe any significant signal in intact planarians hybridized with the antisense DjY1 probe (Fig. 6A). In this respect they appear no different from regenerating planarians hybridized with sense DjY1 RNA as a control (Figs. 6B and 6C).

The results from *in situ* hybridization were compared with Northern blot analysis of RNA extracted from blastemas at different stages of regeneration. Using poly(A)$^+$ RNA, the hybridization signal is seen to be specific, appearing as a single band corresponding to mRNA of the size predicted from the DjY1 cDNA sequence. Although this signal is detected in blastemas at 3 h after cutting, almost as strong a signal is detected in RNA extracted from intact specimens (Fig. 7A). Similar results are obtained when using total RNA extracts except that an extra band becomes discernable (Fig. 7B). Because this extra band is removed by affinity binding of RNA to oligo(dT)-cellulose and is only marginally faster in migration than the poly(A)$^+$ band, it is reasonable to conclude that it represents a population of DjY1 transcripts with shorter poly(A) tails.

Although quantitative changes are difficult to show over the first few days of regeneration, an increase, and then a depletion, of DjY1 transcripts is seen over a longer period. On comparing the levels of DjY1 mRNA from similar amounts of total RNA isolated from 4-and 13-day-old blastemas, it is seen that a higher level of expression occurs in 4 day-old blastemas. (Fig. 7C). The lower level of DjY1 mRNA toward the end of regeneration (13 days) is similar to the basal expression level found in RNA of intact planarians.

An explanation for the absence of an *in situ* hybridization signal in intact specimens, noted above, is probably due to the presence of DjY1 transcripts in cells dispersed throughout the planarian body. A specific localization then occurs in response to injury, that is, as a stress-related event. Neoblasts, distributed throughout the body and already expressing DjY1 transcripts, would first migrate to the site of cutting. Cell proliferation then results in an increase in

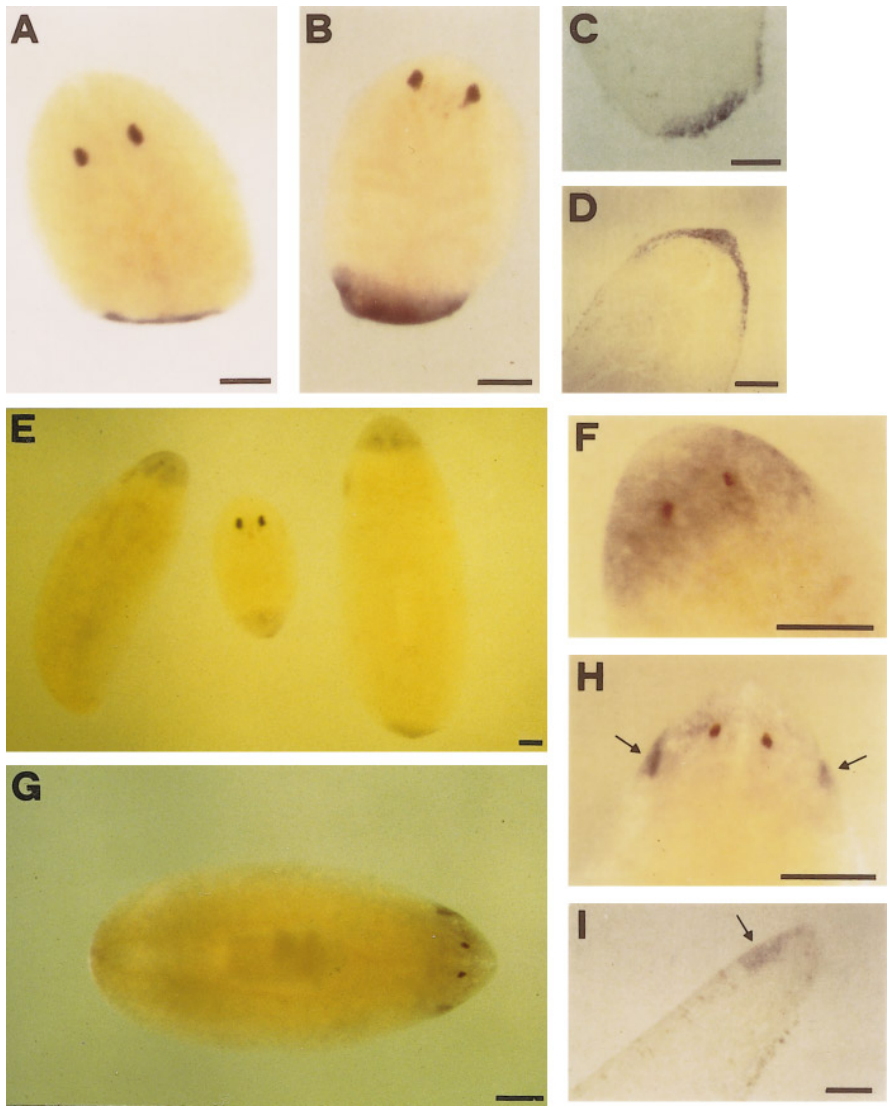

FIG. 5. Expression of DjY1 mRNA during later stages of regeneration, visualized by *in situ* hybridization. (A) Posterior regeneration at 1 day after cutting in whole mount and (B) whole-mount hybridization in 4 day posterior blastema. Wax section preparations of posterior (C) and anterior (D) blastema at 4 days after cutting. (E) Anterior, posterior, and bidirectional regeneration at 6 days after cutting. All the regenerating blastemas show similar level of DjY1 expression. However, the expression level appears decreased compared with that observed in 4-day blastema (B). (F) Higher magnification of 6-day anterior regeneration. (G) Regenerating planarian at 13 days after cutting and higher magnification of its anterior blastema (H). The arrows indicate the auricle regions that show stronger expression of DjY1. (I) longitudinal wax section of whole mount depicted in H, showing stronger expression of DjY1 in the dorsal anterior region (arrow). Scale bars represent 400 μm.

DjY1 transcript levels which are maintained through early differentiation events in the blastema.

Expression of DjY1 Protein during Regeneration

The expression of the DjY1 protein thoughout regeneration was followed by Western blot analysis. Equal amounts of proteins, extracted from blastemas at different regeneration times, were separated by SDS–PAGE and reacted with polyclonal antibodies raised against the GST–DjY1 fusion protein. The immunoblots show specific reaction with a protein doublet with an apparent mass of about 40 kDa. During the course of regeneration, changes were noted in both the absolute abundance and the relative abundance of the two components (Fig. 8). Small amounts of the lower component are detected in extracts from intact planarians

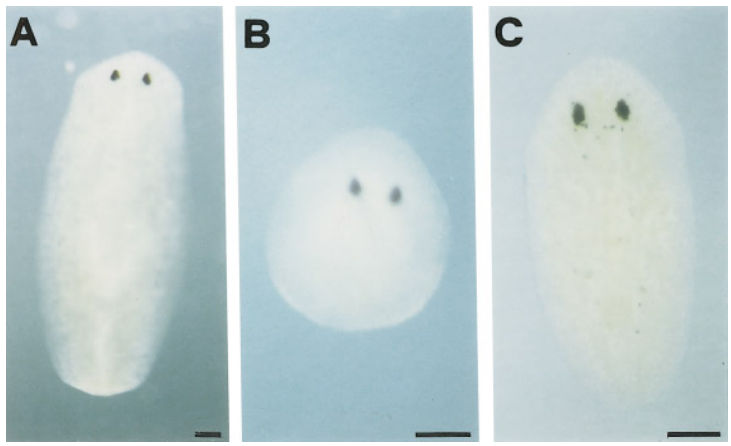

FIG. 6. Specificity of whole-mount *in situ* analysis of DjY1 mRNA. (A) Intact planarian hybridized with antisense RNA probe does not show significant hybridization signal. (B and C) Planarian fragments which are regenerating the tail region at 2 h (B) and at 4 days (C) after cutting, hybridized with sense DjY1 riboprobe. Scale bars represent 400 μm.

and early (1 day) blastemas, but in extracts taken from later stages of regeneration, the upper component becomes the predominant form (Fig. 8). The protein level increases in extracts from 2-day-old blastemas and reaches its peak at 6 days after cutting. From this stage on, as shown for 8-day-old blastemas (Fig. 8), there is a rapid decline in protein level.

Thus the timing of expression of the 40-kDa proteins correlates with the timing of expression of DjY1 mRNA, with up to a 2-day delay between the RNA peak and the protein peak. The discrepancy in protein molecular mass values, of 40 kDa calculated from SDS–PAGE and 30.4 kDa predicted from the cDNA, is quite typical of Y-box proteins and relates to their anomalous electrophoretic properties (Dechamps *et al.*, 1992). The presence of two components of about 40 kDa cross-reacting with antiserum DjY1 could be due to post-translational modification, such as phosphorylation, of the DjY1 protein. If this interpretation is correct, then the upper (phosphorylated) band would be associated with activity during the regeneration process.

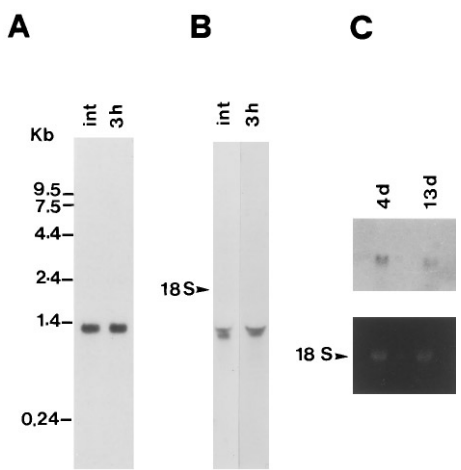

FIG. 7. Northern blot analysis of DjY1 expression. Autoradiograph of a Northern blot of 3.5 μg of poly $(A)^+$ RNA (A) and 30 μg of total RNA (B), prepared from whole bodies of intact planarians (int) and 3 h regenerants (3h), probed with ^{32}P-labeled DjY1 cDNA. Numbers to the left in A are RNA kb markers. 18S indicates the migration position of rRNA in B. (C) RNA prepared from isolated blastemas after 4 days (4d) and 13 days (13d) from cutting. Each lane was loaded with 10 μg of total RNA. Equal loading and integrity of the RNA samples was confirmed in the lower panel by viewing the18S ribosomal RNA (18S) under UV light after staining with ethidium bromide. As with several invertebrates, in planarians is present a characteristic hidden break in the 28S molecule (Zarlenga and Gamble, 1987).

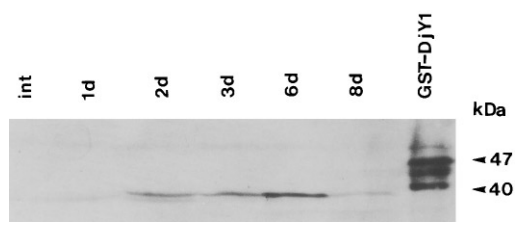

FIG. 8. Western blot analysis of DjY1. Equal amounts of protein extracts (40 μg) from intact (int) planarians and 1-day (1d), 2-day (2d), 3-day (3d), 6-day (6d), and 8-day (8d) isolated blastemas, probed with anti-DjY1 antiserum. GST–DjY1 was used as a control. Like other Y-box proteins, DjY1 migrates more slowly on SDS–PAGE (at 40 kDa) than predicted from the its cDNA sequence.

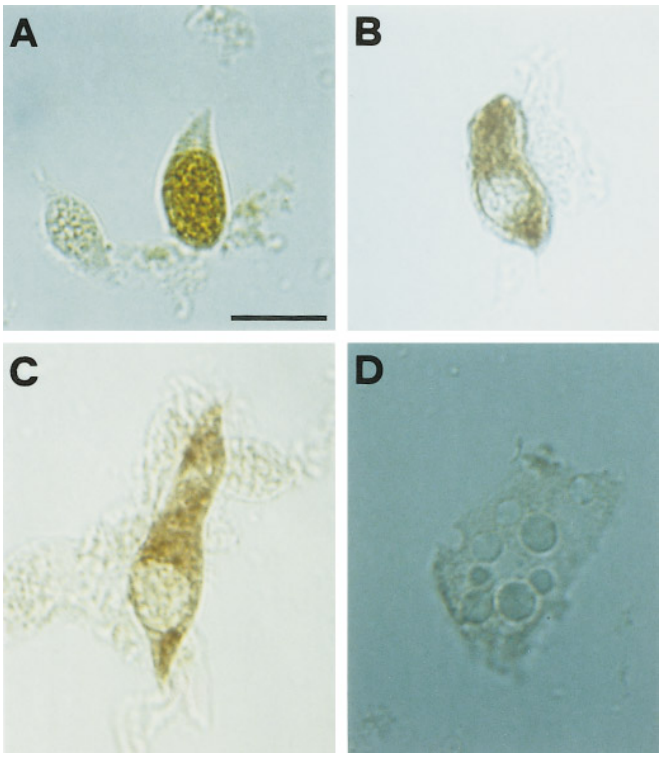

FIG. 9. Dissociated cells immunostained with anti-DjY1 antiserum. (A) Two neoblast-like cells, the nucleus of one showing positive staining. (B and C) Cells at intermediate stages of differentiation, showing positive staining of the cytoplasm. (D) A nonreactive differentiated cell, resembling a gastrodermal cell. Controls omitting the primary (DjY1) antiserum or using a heterologous primary antibody gave no immunostaining (not shown). Scale bar represents 10 μm.

Immunostaining of Blastemal Cells

The DjY1 protein was detected *in situ* by immunostaining preparations of dissociated blastemal cells made at 4, 5, 6, 7, 11, and 15 days after cutting. The general finding is that at any one stage of regeneration DjY1 is seen to be accumulated in only a limited number of cells. However, in preparations from 4- and 5-day-old blastemas, many additional cells showed a low level of immunostaining.

At all stages of regeneration, some of the small, neoblast-like cells showed intense immunstaining in the nucleus (Fig. 9A), whereas other cell types showed immunostaining only in the cytoplasm (Figs. 9B and 9C). The morphology of these latter cells does not resemble any previously described planarian type and it is possible that they are cells in the process of differentiation, being between neoblast and terminally differentiated cell types (Baguñà and Romero, 1981). Clearly identifiable differentiated cells did not show any immunostaining (Fig. 9D). In cell preparations from intact planarians, many fewer immunostained cells were found compared with the regeneration stages.

In Vitro Binding Properties of Recombinant DjY1

The presence of a CSD and RG-repeat motifs predicted from the DjY1 cDNA sequence led us to test the ability of recombinant DjY1 protein (GST–DjY1) to bind RNA *in vitro*. Proteins were separated by SDS–PAGE, transferred to nitrocellulose, and incubated with radiolabeled RNA probes. The results show that GST–DjY1 efficiently binds RNA probes synthesized from cDNAs such as those encoding translation elongation factor 2 (EF2) of *D. japonica* (Fig. 10A) and an oocyte-specific β-tubulin of *Xenopus* (Fig. 10B). The β-tubulin sequence has previously been shown to bind the *Xenopus* Y-box protein FRGY2 (Ladomery and Sommerville, 1994). Bound RNA was still present in the GST–DjY1 bands after washing the transfers extensively in high salt (2.5 M NaCl) (Fig. 10C). As can be seen from Fig. 8, the GST–DjY1 protein is partially hydrolyzed from the carboxyl terminus during expression and extraction, resulting in bands at 44 and 40 kDa in addition to the full-length fusion of 47 kDa. It is interesting to note that only the top two bands bind RNA even after low stringency washing (Fig. 10B) and that only the full-length protein was able to maintain a stable interaction when high stringency (2.5 M NaCl) washing was applied (Fig. 10C). Since all forms of the protein contain the CSD, additional stability of RNA binding must be contributed by sequences in the tail domain. The *Xenopus* Y-box protein, FRGY2, bound the same probes as DjY1, although it showed less stable binding (Figs. 10B and 10C). Various other control proteins, including a nonrelated GST-fusion protein (p54 RNA helicase, Ladomery *et al.*, 1997) and those present in bacterial lysates, failed to bind any detectable level of RNA probe (Figs. 10A and 10B).

To investigate further the binding specificity of DjY1, RNA binding reactions were carried out in solution. Competition assays were set up by adding an excess of unlabeled polynucleotides as competitors, prior to addition of the mRNA sequence probe. Any protein-bound RNA is trapped on nitrocellulose filters and the amount of bound radioactivity is counted. In this type of assay it was again demonstrated that the fusion protein specifically recognizes the EF2 RNA probe. Among the competitors assayed, poly(A, G) competed most effectively for binding to GST–DjY1, but only at a 35-fold excess over the probe (Fig. 10D). All other homopolymers and mixed polymers tested only started to compete at 100- to 1000-fold excess. Probe binding was not competed by double-stranded polynucleotides such as tRNA, poly(dI:dC) and poly(dG:dC), which even improved probe binding (Fig. 10D), presumably by mopping up inhibitors from the reaction mix. Although single-stranded (ss) DNA did compete, again it only did so over the range of 100- to 1000-fold excess, indicating that, even with a complex mixture of sequences, those represented in mRNA molecules are preferred to genomic DNA. Taken together,

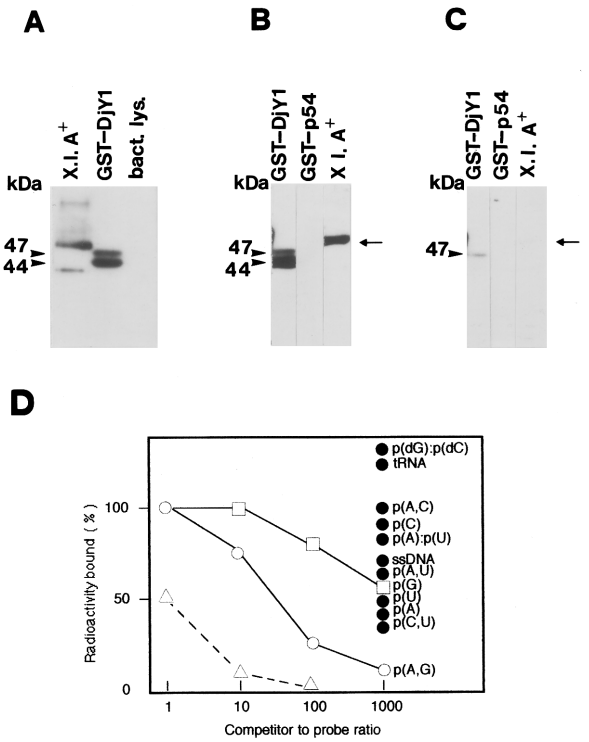

FIG. 10. RNA-binding properties of recombinant DjY1. (A) Transfer of GST-DjY1 binds a ^{32}P-labeled sequence corresponding to the mRNA of *D. japonica* EF2. A poly(A)$^+$ RNP fraction from oocytes of *Xenopus* (X.l. (A)$^+$) is used as a positive control and shows RNA-binding by the oocyte-specific Y-box protein FRGY2. Both GST–DjY1 and a truncated version, lacking ≈3 kDa of protein from the carboxyl end, bind the RNA probe. Bacterial proteins (bact. lys.) do not bind the probe. (B) GST–DjY1 also binds a ^{32}P-labeled probe corresponding to the 3′ terminus of the oocyte-specific β-tubulin mRNA of *Xenopus*. GST–p54 RNA helicase (GST–p54) does not bind the probe. (C) Higher stringency washing (in 2 M NaCl) of the transfer shown in B shows that the full-length GST–DjY1 fusion protein exhibits more stable RNA binding than do either the partially truncated GST–DjY1 or FRGY2. (D) Effect of competing polynucleotides added to the GST–DjY1/riboprobe binding assay in solution. Polynucleotides were added to the protein immediately before the EF2 riboprobe at a competitor to probe mass ratio of 1:1, 10:1, 100:1, and 1000:1. The results are expressed as a percentage of the radioactivity bound to nitrocellulose filters in the absence of added polynucleotide. Two transitions are shown: for poly(A,G) (open circles) and for poly(G) (open squares). The effects of other polynucleotides are shown at 1000:1 excess only (filled circles). The broken line indicates the competition curve expected from a polynucleotide with a protein binding affinity equal to that of the probe.

these results suggest that, *in vitro*, DjY1 binds single-stranded RNA of mixed sequence with a preference for purine residues. In addition to polynucleotide competition, probe binding was partially blocked by addition of anti-DjY1 equivalent to an antiserum dilution of 1:500 (data not shown).

DISCUSSION

DjY1 as an RNA-Binding Protein

The DjY1 protein sequence shows pronounced similarities to the Y-box nucleic acid-binding proteins of other organisms and, in particular, it contains a well-defined cold shock domain (CSD) which is the hallmark of this family of proteins (Wolffe, 1994). The two characteristic consensus sequences RNP1 and RNP2, features of the RNA-binding RRM domain (Burd and Dreyfuss, 1994; Siomi and Dreyfuss, 1997), are also represented in the CSD of DjY1. In addition to the RNP1- and RNP2-like motifs, DjY1 also contains a series of RG repeats spread through its carboxy-tail domain. Such repeats are also found in the two Y-box proteins most similar to DjY1 within the CSD: SmYB, recently identified in the flatworm *S. mansoni* (Franco *et al.*, 1997) and ApY1, a proposed RNA-binding protein identified in the mollusc *A. californica* (Skehel and Bartsch, 1994). The distribution of RG repeats in the tail domains of DjY1, SmYB and ApY1 resembles RGG boxes, defined as closely spaced RGG repeats interspersed with other, often aromatic, residues and found in many RNA-binding proteins (Birney *et al.*, 1993; Burd and Dreyfuss, 1994). DjY1 has 19 RG repeats, of which 1 is RGG; SmYB has 20 RG repeats, of which 3 are RGG; ApY1 has 8 RG repeats, of which 3 are RGG. All 3 proteins have a high density of phenylalanine (F) and tyrosine (Y) residues distributed between blocks of RG repeats (Fig. 3). A similar situation pertains to the single Y-box protein Yps of *D. melanogaster*, which also contains RG repeats in its carboxy-tail domain (Thieringer *et al.*, 1997). We propose that the combination of a CSD with a tail domain containing RG repeats and F/Y-rich spacers constitutes a novel class of RNA-binding proteins, which, so far, have only been described in invertebrates. The contribution of the tail domain of DjY1, in the formation of a stable interaction with RNA, is indicated in the riboprobe binding experiments described in Fig. 10: loss of a 3-kDa fragment from the carboxyl end of the fusion protein results in less stable binding of the riboprobe. It is estimated that the lost fragment contains ten F/Y residues located between two RG blocks.

By analogy to other Y-box proteins used in a developmental context, for instance FRGY2 in *Xenopus* oocytes, regulation of RNA binding may be mediated through reversible phosphorylation (Kick *et al.*, 1987; Braddock *et al.*, 1994; Murray, 1994). Several potential phosphorylation sites are present in DjY1, including three CK2 sites of the type implicated in the regulation of FRGY2. It is not known if these sites are used *in vivo*, the only clue being the transient appearance of an immunoreactive band, which migrates at a rate slightly slower than the native DjY1 protein, during the regeneration process (Fig. 8). It will be interesting to see if this transient component is, indeed, a phosphorylated isoform of DjY1.

The production of DjY1, itself, may also be regulated at a posttranscriptional level. For instance, several sites that

could be involved in the regulation of translation are located in the 3′ UTR of DjY1 mRNA. The presence of a CPE could related to a specific control of translation by altering the poly(A) tail length (Paris and Richter, 1990; Bachvarova, 1992). Moreover, the A+T richness of the 3′ UTR coupled with the presence of two copies of an $A(U)_nA$ sequence, similar to that found in many short-lived mRNAs (Shaw and Kamen, 1986; Jackson and Steinart, 1990), could have a role in the rapid degradation of DjY1 mRNA, once it is no longer required. The use of translational modulation, by means of a variety of posttranscriptional mechanisms, represents an important tool for rapid developmental events, allowing a cell to reply promptly to developmental cues (Curtis *et al.*, 1995). It is likely that selective posttranscriptional controls of gene expression occur in the blastema cells, where a morphogenetic program ensures rebuilding of lost body parts in a short time.

DjY1 Gene Expression during Regeneration

Both the spatiotemporal visualization of transcripts and the increased production of protein clearly implicate DjY1 in planarian regeneration. Although DjY1 is predominantly expressed in the regenerative tissue, the presence of both transcripts and protein in intact planarians, detected in Northern and Western blot analyses, is not unexpected. One of most extraordinary characteristics of the planarian body is the permanent presence of neoblasts, stem cells capable of renewing themselves by cell proliferation, or of becoming committed to a differentiative state, eventually producing all of the various functional cell types. Neoblasts, then, might already be loaded with regulatory gene products in intact planarians, amputation serving to stimulate quantitative, rather than qualitative, changes (Baguñà *et al.*, 1994). Rapid local migration of neoblasts to the wound site, together with cell proliferation, would be sufficient to account for the initial localized hybridization seen in this study. Up-regulation of the DjY1 gene in the growing blastema would be a secondary effect.

Whole-mount RNA hybridizations, performed through the course of regeneration, indicate a biphasic pattern of DjY1 expression. In the first phase, which takes place over the first 4 to 5 days, early expressed DjY1 mRNA is accumulated in the growing blastema and parallels differentiation of important structures, for instance the cerebral ganglia (Coward, 1968) to reach a peak at 4 days. Comparable patterns are observed in the different types of regeneration resulting from the different orientations of cutting. Early DjY1 expression recalls the expression of the Dthox genes, the only other regeneration-related genes visualized in planarians so far (Bayascas *et al.*, 1997). These planarian Hox-related genes, first detected only 1 h after cutting, are expressed in all blastema types, and show differential timing of deactivation.

From day 6 on, the DjY1 mRNA level decreases at a steady rate in all regenerating tissue. Nevertheless, a second phase of DjY1 expression appears in 13-day-old anterior regenerations. This late hybridization signal is restricted to the regions of auricle formation. Therefore, at all stages of regeneration, the highest levels of DjY1 transcripts correspond to both the timing and the location of differentiation events.

A Function for the DjY1 Protein in Morphogenesis

Little is known of how blastema-specific morphogenesis is controlled at the molecular level. Although no other data are available, it is possible that regulatory gene products, Hox proteins for example (Bayascas *et al.*, 1997), are present early in the blastema. Our immunoblotting results show that the amount of DjY1 protein increases within 2 days of regeneration, peaks at 6 days and decreases at 8 days. Therefore, production of the protein product is coincident with localized expression of the DjY1 RNA transcript. These temporal expression data indicate that the DjY1 protein is correlated with differention events.

More detailed information derives from our immunocytological results, which reveal two distinct cellular locations of the DjY1 protein: in the nuclei of a subset of neoblast-like cells, and in the cytoplasm of cells apparently at intermediate stages of differentiation. Detection of DjY1 in the nucleus may well be associated with determination of neoblasts to produce differentiated cell types. It can be hypothesized that nuclear localization represents interaction of DjY1 with gene promoter regions and/or nascent RNA transcripts, both types of interaction having been described for FRGY2 in *Xenopus* oocytes (Gunkel *et al.*, 1995; Sommerville and Ladomery, 1996b; Wolffe and Meric, 1996). Detection of DjY1 in the cytoplasm of differentiating cells might then represent subsequent retention of the protein on mRNA, perhaps to regulate translation of differentiation-specific messages, again, a function commonly attributed to Y-box proteins (discussed below). We suggest that the cytoplasmic-staining cells are at an intermediate stage of differentiation, because we cannot classify them, unambiguously, as any of the types previously described, in spite of the fact that the maceration technique employed makes cell identification easy (Baguñà and Romero, 1981).

Although high levels of DjY1 mRNA are found in the regenerative blastema, only a limited number of cells appear to express large amounts of the protein. It is possible that all of the blastemal cells contain DjY1 mRNA, but that only some cells activate its translation and thereafter enter a differentiation pathway. Many additional cells from early blastemas are immunostained weakly and it may be that the different levels of staining reflect cell populations undergoing non-synchronous differentiation.

The evidence that recombinant DjY1 efficently binds RNA *in vitro* supports our claim that the native protein is involved in mechanisms of posttranscriptional regulation of regeneration-related genes. The notion of Y-box proteins as regulatory molecules is well established (Sommerville and Ladomery, 1996a,b; Wolffe and Meric, 1996). The

mammalian Y-box protein p50 is found bound to mRNA in a range of somatic cell types (Evdokimova *et al.*, 1995) and p50 transient transfection in COS cells produces drastic inhibition of translation. Yet, low concentrations of p50 remain bound to mRNA in polyribosomes, suggesting that it is the reduction in number of p50 molecules per mRNA molecule that determines the transition from inactive to active states (Davydova *et al.*, 1997). The maternal mRNA in *Xenopus* oocytes is masked from translation in particles containing high concentrations of the Y-box protein FRGY2 (Sommerville and Ladomery, 1996b) and *in vivo* expression of FRGY2 along with reporter transcripts leads to translation repression (Bouvet and Wolffe, 1994; Braddock *et al.*, 1994). Recently, it has been demonstrated that the heterochronic RNA-binding protein LIN-28 also contains a CSD and so would be classed as a Y-box protein (Moss *et al.*, 1997). LIN-28 belongs to a hierarchy of regulatory elements involved in developmental timing of *Caenorhabditis*, and acts as a switch that controls choices of stage-specific fates (Moss *et al.*, 1997).

So how would the proposed activity of DjY1 in regulating translation during regeneration relate to morphogenetic events? Morphogenesis begins early in the planarian blastema: head determination occurs within a few hours of cutting and the beginning of cell differentiation is timed at 1–2 days (Ghirardelli *et al.*, 1988; Baguñà *et al.*, 1994; Cebriá *et al.*, 1997). By 4–6 days of regeneration, many differentiation events have been completed (Baguñà *et al.*, 1994). Accordingly, we observed the presence of well formed eyespots in 5- to 6-day-old cephalic regenerations of *D. japonica*. Within this scenario, we suggest that DjY1 acts as a chaperone for differentiation-specific mRNAs, maintaining them in a single-stranded configuration through a pathway that might include short-term storage, efficient translation at the appropriate time, and eventual degradation (Beelman and Parker, 1995; Curtis *et al.*, 1995). In this context, the nuclear presence of DjY1 protein may be required to efficiently direct RNA transcripts into a translational regulatory pathway, as has been demonstrated for other Y-box proteins (Bouvet and Wolffe, 1994; Braddock *et al.*, 1994). Timing cues for stage-specific events of regeneration would then be provided by other factors, for instance protein kinases or phosphatases to alter the stability of interaction of DjY1 with mRNA, or more specialized RNA-binding proteins to determine message-specific translation.

ACKNOWLEDGMENTS

We are grateful to Dr. Hidefumi Orii (Himeji Institute of Technology, Japan) for the generous gift of the *D. japonica* cDNA bank and for providing us with the planarian GI clonal strain and EF2 cDNA. This research was supported by the Italian Department of Education (MURST), the Wellcome Trust (to J.S.) and a short term EMBO fellowship (to A.S.).

REFERENCES

Bachvarova, R. F. (1992). A maternal tail of poly(A): The long and the short of it. *Cell* **69,** 895–897.

Baguñà, J., Salò, E., Romero, R. R., Garcia-Fernàndez, J., Bueno, D., Muñoz-Marmol, A., Bayascas-Ramirez, J. R., and Casali, A. (1994). Regeneration and pattern formation in planarians: Cell, molecules and genes. *Zool. Sci.* **11,** 781–795.

Baguñà, J., and Romero, R. R. (1981). Quantitive analysis of cell types during growth, degrowth and regeneration in the planarian *Dugesia mediterranea* and *Dugesia tigrina*. *Hydrobiologia* **84,** 181–194.

Bayascas, J. R., Castillo, E., Muñoz-Màrmol, A. M., and Salo′ E. (1997). Planarian Hox genes: Novel patterns of expression during regeneration. *Development* **124,** 141–148.

Beelman, C. A., and Parker, R. (1995). Degradation of mRNA in eukaryotes. *Cell* **81,** 179–183.

Birney, E., Kumar, S., and Krainer, A. R. (1993). Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors. *Nucleic Acids Res.* **21,** 5803–5816.

Bouvet, P., and Wolffe, A. P. (1994). A role for transcription and FRGY2 in masking maternal mRNA within *Xenopus* oocytes. *Cell* **77,** 931–941.

Braddock, M., Muckenthaler, M., White, M. R. H., Thorburn, A. M., Sommerville, J., Kingsman, A. J. and Kingsman S. M. (1994). Intron-less RNA injected into the nucleus of *Xenopus* oocytes accesses a developmentally-regulated translation control pathway. *Nucleic Acids Res.* **22,** 5225–5264.

Bueno, D., Espinosa, L., Soriano, M. A., Battle, E., Baguñà, J., and Romero, R. R. (1995). TCEN-49, a monoclonal antibody that identifies a central body antigen in the planarian *Dugesia* (*Girardia*) *tigrina*. Implications for pattern formation and positional signalling mechanisms. *Hydrobiologia* **305,** 235–240.

Burd, C. G., and Dreyfuss, G. (1994). Conserved structures and diversity of functions of RNA-binding proteins. *Science* **265,** 615–621.

Cebriá, F., Vispo, M., Newmark, P., Bueno, D., and Romero, R. (1997). Myoocyte differentiation and body wall muscle regeneration in the planarian *Girardia tigrina*. *Dev. Genes Evol.* **207,** 306–316.

Coward, S. J. (1968). Effects of actinomycin on regeneration give evidence for sequential gene activation. *Nature* **219,** 1257–1258.

Curtis, D., Lehmann, R., and Zamore, P. D. (1995). Translational regulation in development. *Cell* **81,** 171–178.

Davydova, E. K., Evdokimova, V. M., Ovchinnikov, L. P., and Hershey, J. W. B. (1997). Overexpression in COS cells of p50, the major core protein associated with mRNA, results in translation inhibition. *Nucleic Acids Res.* **25,** 2911–2916.

Deschamps, S., Viel, A., Garrigos, M., Denis, H., and Le Maire, M. (1992). mRNP4, a major mRNA-binding protein from *Xenopus* oocytes is identical to transcription factor FRGY2. *J. Biol. Chem.* **267,** 13799–13802.

Evdokimova, V. M., Wei, C. L., Sitikov, A. S., Simonenko, P. N., Lazarev, O. A., Vasilenko, K. S., Usinov, V. A., Hershey, J. W., and Ovchinnikov, L. P. (1995). The major protein of messenger ribonucleoprotein particles in somatic cells is a member of the Y-box binding transcription factor family. *J. Biol. Chem.* **270,** 3186–3192.

Farnesi, R. M., and Tei, S. (1980). *Dugesia lugubris* s.l. auricles: Research into the ultrastructure and on the functional efficiency. *Riv. Biol.* **73,** 65–77.

Franco, G. R., Garratt, R. C., Tanaka, M., Simpson, A. J., and Pena, S. D. (1997). Characterization of a *Schistosoma mansoni* gene encoding a homologue of the Y-box binding protein. *Gene* **198,** 5–16.

Ghirardelli, E., Marzari, R., Ferrero, E. A., and Bittolo, M. (1988). Determination and differentiation in planarian regeneration: Molecular aspects of blastema heterogeneity. *In* "Free-Living and Symbiontic Plathelminthes" (Ax/Ehlers/Sopott-Ehlers, Eds-.),Vol. 36, pp. 79–84. Fisher Verlag, Stuttgart–New York.

Gremigni, V., and Miceli, C. (1980). Cytophotometric evidence for cell "transdifferentiation" in planarian regeneration. *Wilhelm. Roux's Arch.* **188,** 107–113.

Gunkel, N., Braddock, M., Thorburn, A. M., Muckenthaler, M., Kingsman, A. J., and Kingsman, S. M. (1995) Promoter control of translation in *Xenopus* oocytes. *Nucleic Acids Res.* **23,** 405–412.

Jackson, R. J., and Steinart, N. (1990). Do the poly(A) tail and the 3′ untranslated region control mRNA translation? *Cell* **62,** 15–24.

Jiang, W., Hou, Y., and Inouye, M. (1997). CspA, the major cold-shock protein of *Escherichia coli* is an RNA chaperone. *J. Biol. Chem.* **272,** 196–202.

Kick, D., Barrett, P., Cummings, A., and Sommerville, J. (1987). Phosphorylation of a 60 kDa polypeptide from *Xenopus* oocytes blocks messenger RNA translation. *Nucleic Acids Res.* **15,** 4099–4109.

Kingsley, P. D., and Palis, J. (1994). GRP2 proteins contain both CCHC zing fingers and a cold shock domain. *Plant Cell* **6,** 1522–1523.

Kozak, M. (1987). An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15,** 8125–8148.

Ladomery, M., and Sommerville, J. (1994). Binding of Y-box proteins to RNA: involvement of different protein domains. *Nucleic Acids Res.* **22,** 5582–5589.

Ladomery, M., Wade, E., and Sommerville, J. (1997). Xp54, the *Xenopus* homologue of human RNA helicase p54, is an integral component of stored mRNP particles in oocytes. *Nucleic Acids Res.* **25,** 965–973.

Landsman, D. (1992). RNP-1, an RNA-binding motif is conserved in the DNA-binding cold shock domain. *Nucleic Acids Res.* **20,** 2861–2864.

Marello, K., LaRovere, J., and Sommerville, J. (1992). Binding of *Xenopus* oocyte masking proteins to mRNA sequences. *Nucleic Acids Res.* **20,** 5593–5600.

Matsumoto, K., Meric, F., and Wolffe, A. (1996). Translational repression dependent on the interaction of the *Xenopus* Y-box protein FRGY2 with mRNA. *J. Biol. Chem.* **271,** 22706–22712.

Montgomery, J. R., and Coward, S. J. (1974). On the minimal size of a planarian capable of regeneration. *Trans. Am. Microsc. Soc.* **13,** 364–397.

Moss, E., Lee, R. C., and Ambros, V. (1997). The cold shock domain protein LIN-28 controls developmental timing in *C. elegans* and is regulated by the lin-4 RNA. *Cell* **88,** 637–646.

Murray, M. T. (1994). Nucleic acid binding properties of the *Xenopus* oocyte Y-box protein mRNP3+4. *Biochemistry* **33,** 13910–13917.

Murray, M. T., Schiller, D. L., and Franke, W. W. (1992). Sequence analysis of cytoplasmic mRNA-binding proteins of *Xenopus* oocytes identified a family of RNA-binding proteins. *Proc. Natl. Acad. Sci. USA* **89,** 11–15.

Orii, H., Agata, K., and Watanabe, K. (1993). POU-domain genes in planarian *Dugesia japonica:* The structure and expression. *Biochem. Biophys. Res. Commun.* **192,** 1395–1402.

Oubridge, C., Ito, N., Evans, P. R., Teo, C. H., and Nagai, K. (1994). Crystal structure at 1.92 A resolution of the RNA-binding domain of the U1A spliceosomal protein complexed with an RNA hairpin. *Nature* **372,** 432–438.

Paris, J., and Richter, J. D. (1990). Maturation-specific polyadenilation and translational control: Diversity of cytoplasmic polyadenylation elements, influence of poly(A) tail size, and formation of stable polyadenylation complexes. *Mol. Cell. Biol.* **10,** 5634–5645.

Rokeach, L. A., Haselby, J. A., and Hoch, S. O. (1988). Molecular cloning of a cDNA encoding the human Sm-D autoantigen. *Proc. Natl. Acad. Sci. USA* **85,** 4832–4836.

Schindelin, H., Jiang, W., Inouye, M., and Heinemann, U. (1994). Crystal structure of CspA, the major cold shock protein of *Escherichia coli. Proc. Natl. Acad. Sci. USA* **91,** 5119–5123.

Shaw, G., and Kamen, R. (1986). A conserved AU sequence from the 3′ untraslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46,** 659–667.

Skehel, P. A., and Bartsch, D. (1994). Characterization of a Y-box factor from *Aplysia californica. Gene* **145,** 231–235.

Siomi, H., and Dreyfuss, G. (1997). RNA-binding proteins as regulators of gene expression. *Curr. Opin. Genet. Dev.* **7,** 345–353.

Sommerville, J., and Ladomery, M. (1996a). Masking of mRNA by Y-box proteins. *FASEB J.* **10,** 435–443.

Sommerville, J., and Ladomery, M. (1996b). Transcription and masking of mRNA in germ cells: Involvement of Y-box proteins. *Chromosoma* **104,** 469–478.

Suzuki, K., Olvera, J., and Wool, I. G. (1991). Primary structure of rat ribosomal protein S2. A ribosomal protein with arginine-glycine tandem repeats and RGGF motifs that are associated with nucleolar localization and binding to ribonucleic acids. *J. Biol. Chem.* **226,** 20007–20010.

Thieringer, H. A., Singh, K., Trivedi, H., and Inouye, M. (1997). Identification and developmental characterization of a novel Y-box protein from *Drosophila melanogaster. Nucleic Acids Res.* **25,** 4764–4770.

Wolffe, A. P. (1994). Structural and functional properties of the evolutionary ancient Y-box family of nucleic acid binding proteins. *BioEssay* **16,** 245–251.

Wolffe, A. P., and Meric, F. (1996). Coupling transcription to translation: A novel site for the regulation of eukaryotic gene expression. *Int. J. Biochem. Cell Biol.* **28,** 247–257.

Wolffe, A. P., Tafuri, S., Rajan, M., and Familiari, M. (1992). The Y-box factors: A family of nucleic acid binding proteins conserved from *Escherichia coli* to man. *New Biol.* **4,** 290–298.

Zarlenga, D. S., and Gamble, H. R. (1987). Simultaneous isolation of preparative amounts of RNA and DNA from *Trichinella spiralis* by cesium trifluoroacetate isopicnyc centrifugation. *Anal. Biochem.* **162,** 569–574.

Received for publication March 19, 1998
Revised May 26, 1998
Accepted June 17, 1998